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# In Vitro Response of Guinea Pig Peritoneal Macrophages to *Legionella pneumophila*

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Transmission and scanning electron microscopy were used to study the phagocytosis of virulent and avirulent strains of *Legionella pneumophila*. The interaction between *L. pneumophila* and peritoneal macrophages from normal guinea pigs or from animals that had survived infection was studied. The virulent strains survived and proliferated within the phagocyte after ingestion by either type of macrophage, whereas the avirulent strain of bacteria was killed by normal macrophages. Although the addition of immune serum enhanced phagocytosis, the outcome was the same as with normal serum.

The mechanisms of immunity against *Legionella pneumophila* (2), the causative agent of Legionnaires disease, have not been well defined. Many defense mechanisms are available to the host, but one of the most important primary defenses in humans is a phagocytic cell, the macrophage. The role of macrophages in immunological and inflammatory responses to many kinds of bacterial infection has been well documented (13).

The purpose of this study was to investigate the phagocytic and bactericidal capabilities of macrophages when exposed to *L. pneumophila*. The interaction between peritoneal macrophages from normal and previously infected and convalescing guinea pigs, and virulent and avirulent strains of *L. pneumophila* was studied by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

## MATERIALS AND METHODS

**Bacteria.** The strains of *L. pneumophila* (serogroup I) employed were obtained from the Centers for Disease Control, Atlanta, Ga. The virulent Philadelphia-1 and Washington strains (human lung isolates) and an avirulent Philadelphia-1 stram were obtained as yolk sac suspensions (passage unknown). Before use, the virulent strains were passed three times in embryonated chicken yolk sac, and the avirulent strain was passed three times on Mueller-Hinton agar. The intraperitoneal 50% lethal dose in outbred Hartley strain guinea pigs was determined to be $10^6$ organisms of the virulent Washington strain and $3.5 \times 10^6$ organisms of the virulent Philadelphia-1 strain. A challenge dose of $10^8$ of an avirulent variant of the Philadelphia-1 strain did not produce death in guinea pigs. The virulent Washington and avirulent Philadelphia 1 strains were cultured on Mueller-Hinton agar supplmented with 2% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) and 1% hemoglobin and incubated at 37°C for 48 h in a humid atmosphere of air containing 5% $CO_2$. The virulent Philadelphia-1 strain was grown on charcoal-yeast agar (5). Organisms were scraped off the plates, washed once with Hanks balanced salt solution, and suspended in sufficient Earle 199 medium supplemented with 10% normal or immune (1:128 microagglutinin titer) guinea pig serum to contain approximately $10^8$ organisms per ml.

**Peritoneal macrophages.** Outbred Hartley strain guinea pigs (Buckberg Lab Animals, Tompkins Cove, N.Y.), weighing approximately 400 to 500 g, were injected with 20 ml of 1.5% sodium caseinate (BBL Microbiology Systems, Cockeysville, Md.). Four days later, the peritoneal exudate cells (approximately 75% macrophages) were harvested and processed by the method of David et al. (4). Macrophages were cultured on petri dishes or in Leighton tubes at 37°C for 2 h as previously described (10, 11).

**Cell culture infection.** Nonadherent cells of peritoneal cell culture preparations were removed with two washes of Hanks balanced salt solution. Bacteria, suspended in Earle 199 medium containing either 10% normal or immune guinea pig serum, were then added to macrophage cultures at a ratio of 100 organisms to 1 macrophage and incubated at 37°C for 2 h. The inoculum was then removed from the macrophage cultures and the cells were washed three times with Hanks balanced salt solution. Macrophage suspensions obtained from guinea pigs 7, 10, 16, or 30 days after infection were infected in the same manner, using autologous immune serum.

Macrophages to be examined at 2 h were processed at this time for TEM and SEM. Fresh medium was added to cultures which were held longer to minimize additional phagocytosis and reincubated at 37°C. Antibiotics were not used. The mean percentage of macrophages containing ingested bacteria and the subsequent fate of ingested organisms were calculated from

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four replicate determinations at each sampling time. Approximately 50 cells were examined in each experiment.

**TEM.** Infected macrophages were processed for examination by TEM as previously described (10). For comparison, uninfected macrophages were processed simultaneously. All preparations were examined with a Hitachi HU-12 electron microscope operating at 75 kV.

**SEM.** Infected cover slips were fixed for 1 h at 4°C in 2% glutaraldehyde adjusted to pH 7.4 with 0.1 M cacodylate buffer. Cover slips were washed with buffer, placed in 1% osmium tetroxide in 0.1 M phosphate (pH 7.2) for 1 h, dehydrated in a series of 10 to 100% ethanol washes, and dried under carbon dioxide by the critical-point method (14). Dried specimens were sputter-coated with gold (10 nm). Observations were made in a JEOL 100B electron microscope modified with a scanning accessory. SEM specimens were viewed at an accelerating voltage of 40 kV. Photographs were made on Polaroid film (55 N/P) at a scan speed of 50 s.

## RESULTS

**Phagocytosis of *L. pneumophila*.** The virulent and avirulent Philadelphia-1 and Washington strains were not readily phagocytized by normal macrophages. After the 2-h interaction period, preparations viewed by TEM indicated that less than 10% of the macrophages contained a few organisms which appeared within a membrane-bound cytoplasmic vesicle (Fig. 1). Macrophages from previously infected animals were more actively phagocytic; approximately 50% of the phagocytes contained one to three organisms per phagocyte. The addition of immune serum to suspensions of the virulent strains before interaction with phagocytes made them more susceptible to phagocytosis by both types of macrophages. In these instances, almost all of the macrophages contained 5 to 10 bacteria per cell. In similar preparations viewed by SEM after the 2-h interaction period, the virulent organisms either were seen on the surface of less than 10% of the cells or were being ingested by the cell (Fig. 2a). Phagocytic engulfment of bacteria was not a predominant observation, and the macrophages did not appear to be stimulated or attracted to the presence of bacteria on the surface of the cover slip. Four hours after inoculation of Leighton tubes, macrophages were either flat with a raised central portion or rounded with numerous microprojections (Fig. 2b).

**Fate of ingested *L. pneumophila*.** Virulent organisms replicated within the macrophages regardless of the immune status of serum or macrophages. Many macrophages contained distended vacuoles filled with organisms (Fig. 3); bacteria undergoing binary fission were occasionally seen.

Numerous bacteria were observed on the surface of macrophages 24 h later; the appearance of the cell membrane indicated disruption of the macrophages and subsequent release of organisms. Both rod-shaped and coccoid forms were seen on the macrophage surfaces (Fig. 4).


FIG. 1. *Normal macrophages in normal serum 1 h after inoculation with the virulent L1 strain of L. pneumophila. Ingested organisms are in cytoplasmic vesicles.*

In contrast, the avirulent Philadelphia-1 strain was killed by normal macrophages; no intact organisms were observed 1 day after infection. Organisms appeared degraded with increased density of the cell matrix and loss of integrity of cell walls.

We have obtained similar results on phagocytosis and intracellular fate of *L. pneumophila* by light microscopy (unpublished data).

## DISCUSSION

Our studies by TEM and SEM demonstrated that both virulent and avirulent strains of *L. pneumophila* are phagocytized by guinea pig peritoneal macrophages in vitro. This finding is consistent with that reported by Johnson et al. (8). The ultimate fate of these organisms differed depending upon virulence. Replication of virulent strains within phagosomes of macrophages was observed by TEM; coccoid as well as rod-shaped organisms were seen on the surface of macrophages 24 h later by SEM. The phagocytic uptake and ultimate replication of the virulent

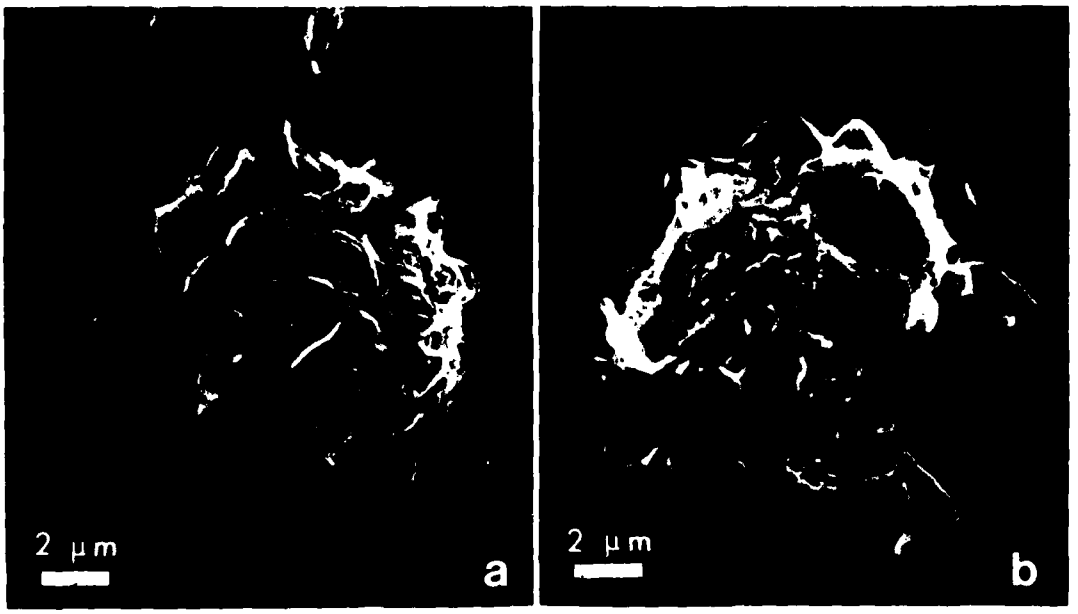


FIG. 2. *Scanning electron micrographs of peritoneal macrophages in normal serum. Coccoid (arrowhead) and bacillary (arrows) forms of L. pneumophila remain on the surface of a macrophage (a) 1 h after inoculation. A normal macrophage in normal serum for the same length of time without bacteria is shown for comparison (b).*

Philadelphia-1 and Washington strains within peritoneal macrophages were similar to those reported with alveolar macrophages obtained from normal cynomolgus monkeys (10).

We observed that the avirulent strain was destroyed by macrophages after ingestion. The loss of virulence may be associated with loss of ability of the organism to survive within macrophages.

The difference in the fate of the host may in part be due to the outcome of the interaction between macrophages and organisms. The avirulent variant of *L. pneumophila* is killed and degraded by macrophages, and death is averted.

The ability of immune serum to opsonize microorganisms, leading to the ultimate destruction of the agent, has been well established. In our study, as with that of Johnson et al. (8), pretreatment of *L. pneumophila* with specific immune serum enhanced uptake by macrophages. However, we demonstrated that immune serum did not potentiate the killing of organisms.

In our study, macrophages obtained from previously infected guinea pigs were of no consequence in the ultimate outcome of the macrophage-bacterium relationship. Although macrophages from convalescing animals were more avid in phagocytosis of bacteria, neither phagocytes alone nor a combination of phagocytes with immune serum resulted in destruction of ingested organisms. It is possible that soluble lymphokines, e.g., macrophage inhibition factor or sensitized lymphocytes, may play a role in the ultimate destruction of bacteria within macrophages. It is not known what mechanisms are

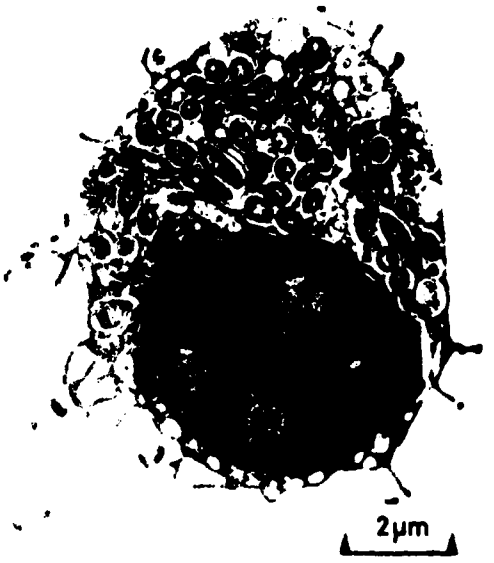


FIG. 3. *Macrophage from a previously infected guinea pig in normal serum 24 h after inoculation with the Washington strain of L. pneumophila. The cytoplasm is filled with organisms.*

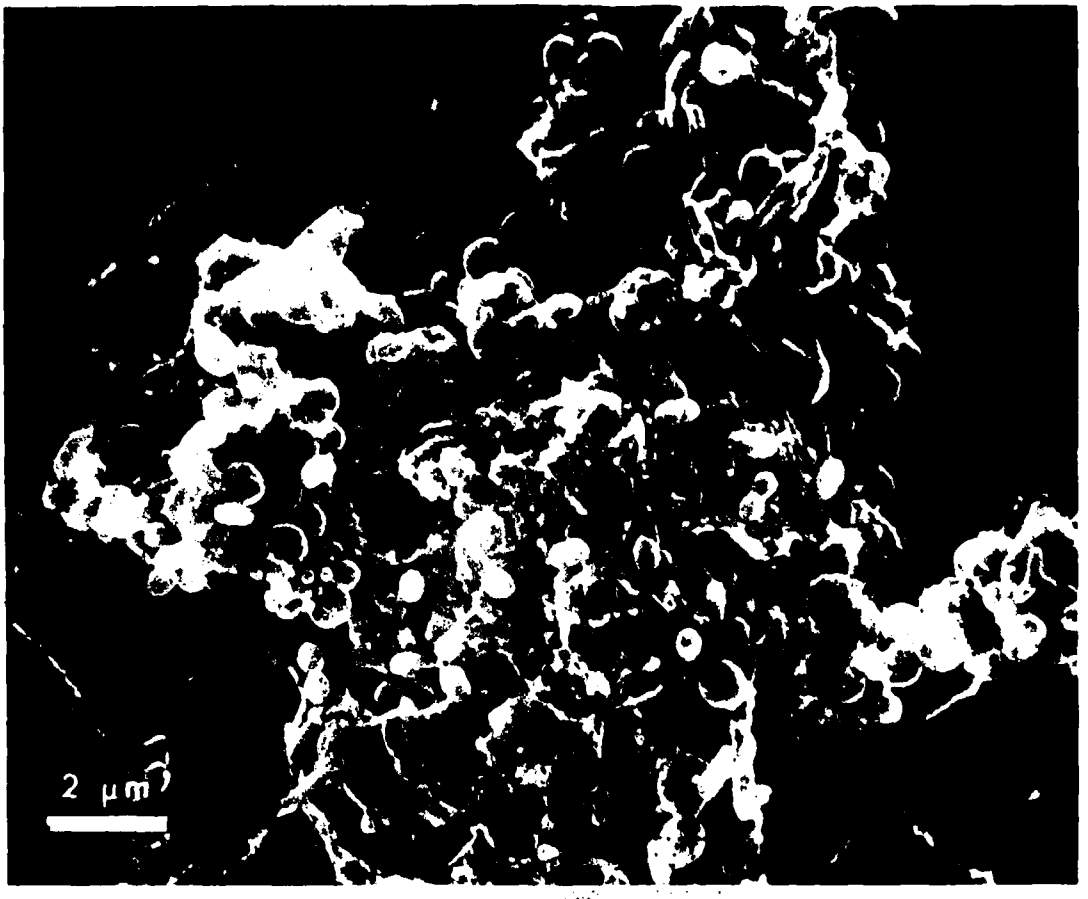


FIG. 4. *Scanning electron micrograph of a normal macrophage in normal serum 24 h after inoculation with the Washington strain of L. pneumophila. Bacteria have multiplied within this macrophage and killed it. Numerous coccoid and bacillary organisms adhere to the cytoplasmic debris.*

involved in the replication of the virulent Philadelphia-1 and Washington strains within macrophages. Microorganisms that are ingested by phagocytes are usually enclosed within well-defined phagosomes. The normal sequence is the fusion of lysosomes with the phagosomes, thus exposing the microorganisms to lysosomal enzymes, leading to destruction of the bacteria. The ability of the virulent strains to avoid destruction within the phagosomes of macrophages may be due to the absence of lysosomal fusion, as has been reported with mycobacteria (1, 6), or possibly resistance of virulent bacteria to the lysosomal enzymatic digestion, as reported with protozoa (3, 7, 9) and chlamydiae (12).

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